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14. ABSTRACT

A central tenet of the field of bone metastases is that the bone microenvironment supplies factors, such as TGF-beta, stimulating prostate cancer cell signaling and altering their phenotype. TGF-beta signaling in cancer is however complex and can lead to the activation of numerous genes. We have identified many of these genes by microarray analysis and have validated the gene reported here. Of these, PMEPA1 as the most highly upregulated gene. We have cloned the PMEPA1 promoter and full-length gene and have begun promoter analysis of the TGFβbetaresponse element. We are in the process of overexpressing PMEPA1 in prostate cancer lines. In vivo experiments are in progress to determine the effect of a TGFbeta RI kinase inhibitor, SD-208, on the development and progression of prostate cancer metastases to bone due to PC-3, LuCAP and C42B prostate cancers.

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Introduction

Prostate cancer has a propensity to grow in the skeleton and cause significant morbidity. Once housed in bone, prostate cancer is incurable. Bone is a rich storehouse of growth factors, which stimulate signaling in metastatic cancer cells. Bone-derived TGFβ increases tumor secretion of factors that activate bone remodeling, fueling a vicious cycle, which drives the growth and survival of prostate bone metastases. In prostate cancer cells, TGFβ signals through two receptor subunits and, further downstream, p38 MAP kinase. Hypothesis: *TGFβ mediates prostate cancer metastases to bone via p38 MAP kinase pathway. TGFβ and/or p38MAP kinase signaling inhibitors will reduce the development and progression of prostate cancer bone metastases to bone.* Two orally active inhibitors of these serine/threonine kinases will be tested in an animal model of prostate cancer bone metastases. We propose three Specific Aims. Aim 1: To test a TGFβRI kinase inhibitor and a p38 MAPK inhibitor against three human prostate cancer models of skeletal metastasis in mice. Aim 2: To determine the molecular targets of these inhibitors in prostate cancer cells *in vitro* and test their impact on tumor growth and bone metastases *in vivo*. Aim 3: To test the efficacy of combined TGFβRI and p38 MAP kinase inhibitors against three prostate cancer models *in vivo*.

Body

BACKGROUND. The skeleton is a major site of metastasis by advanced prostate cancer. Last year 220,900 cases of prostate cancer were diagnosed in the United States, where it is now the most commonly diagnosed cancer and the second most common cause of cancer mortality in men, with 28,900 deaths (Crawford, 2003). One fourth of diagnosed patients will die from the disease, the majority of them with metastases to the skeleton. Once cancer becomes housed in bone, it is incurable. The average survival from time of diagnosis of skeletal metastases in prostate cancer patients is 40 months. When prostate tumor cells metastasize to the skeleton, the most common response is osteoblastic: characterized by net formation of disorganized new bone, which results in fractures, severe and intractable bone pain, and nerve compression. Metastasis to bone thus causes prolonged, serious morbidity for many prostate cancer patients. Treatment to prevent or halt the progression of bone metastases (Reddi et al, 2003; O'Keefe and Guise, 2003). would increase survival and improve quality of life for men with prostate cancer

Transforming growth factor-β in cancer is a two-edged sword. TGFβ is a growth inhibitor and a tumor suppressor at early stages of the oncogenic cascade. However, advanced cancers often lose the growth inhibition by TGFβ but continue to respond to the factor. The net effect is that TGFβ is a metastasis enhancer for advanced cancers. Since bone is a major source of active TGFβ, the factor plays a crucial role in the vicious cycle of bone metastases. Blockade of the TGFβ pathway effectively decreases metastases in several animal models (Yin et al, 1996; Muraoka et al, 2002; Yang et al, 2002).

Transforming growth factor-β in bone is released from mineralized matrix in active form by osteoclastic resorption (Dallas et al, 2002), which is very prominent in prostate cancer metastases. TGFβ acts on tumor cells to increase the secretion of factors that inappropriately stimulate bone cells (Chirgwin & Guise, 2003a,b). The interactions

between bone and cancer constitute a vicious cycle, which enhances skeletal metastases (Mundy, 2002). Extensive data show that TGFβ is a major bone-derived factor responsible for driving the vicious cycle of cancer metastases in bone. TGFβ increases tumor secretion of factors such as endothelin-1, IL-6, IL-11, PTHrP, and VEGF. These factors stimulate both osteoblastic synthesis of disorganized new bone and osteolytic destruction of the skeleton adjacent to tumor cells. The cellular and molecular components of the vicious cycle between tumor and bone offer opportunities for therapeutic intervention to decrease skeletal metastases (Coleman, 2002; Guise & Chirgwin, 2003a). TGFβ in particular is an important target for intervention against prostate cancer skeletal metastases.

Therapy to block TGFβ signaling in bone metastases. Previous work has demonstrated the effectiveness of TGF-beta inhibition to decrease metastases, but these experiments have used protein-based treatment or ex vivo manipulations of the tumor cells (Yin et al, 1996; Muraoka et al, 2002; Yang et al, 2002). Orally active small-molecule inhibitors of the TGFβ pathway would be much more practical. This proposal will test two inhibitors of serine/threonine kinases. The first directly targets the TGFβ receptor kinase. The second targets p38 MAP kinase, which is a major downstream effector of TGFβ signaling in cancer cells. Both targets are serine/threonine kinases. Our preliminary data show that inhibition of TGFβ signaling is effective in an animal model of cancer bone metastases. The work proposed will test the two serine/theronoine kinases inhibitors in animal models of human prostate cancer in bone: one in which the response is osteolytic, two others in which it is osteoblastic. The experiments proposed will rapidly provide the preclinical data necessary for these two drugs to be placed in clinical trails for prostate cancer bone metastases.

Hypotheses: 1) TGFβ mediates prostate cancer metastases to bone via p38 MAP kinase. Specific serine/threonine kinase small-molecule inhibitors of the type I TGFβ receptor kinase and of p38 MAP kinase will reduce the development and progression of prostate cancer metastases to bone, due to either osteoblastic or osteolytic diseases. 2) Orally active inhibitors of these serine/threonine kinases will be effective in animal models of prostate cancer bone metastases to decrease metastases and tumor burden and to increase survival. 3) The two drugs may be more effective in combination than singly, if p38 MAP kinase also mediates TGFβ-independent metastatic functions. 4) Specific targets of TGFβ signaling in prostate cancer cells contribute directly to the bone phenotype of metastases. One such factor may be the type I membrane protein PMEPA1, which is regulated by TGFβ and expressed by prostate cancers. 5) Expression of PMEPA1 on the surface of cancer cells will increase the development and progression of prostate cancer metastases to bone.

Specific Aim 1: To determine the effect of TGFβRI kinase or p38 MAPK blockade separately against 3 human prostate cancer models of skeletal metastasis in mice (hypotheses 1 & 2).

Results and Progress: This experiment is in the final stages of progress with respect to the TGFβRI kinase inhibitor and the p38MAP kinase inhibitor. We have tested the effects of the TGFβRI kinase, SD-208, on the development and progression of bone

metastases due to PC-3, LuCAP23.1 and C42B prostate cancer. This aim has taken longer than originally planned because we had to determine long-term pharmacokinetics for drug delivery in the food. We have pharmacokinetic data that 50 and 100 mg/kg of SD-208 added to food result in drug levels that were effective in our mouse model of breast cancer metastases to bone. Here we show data that TGFβRI kinase, SD-208, reduced osteolytic bone metastases due to PC-3, but increased osteoblastic bone metastases due to LuCAP23.1. There was no effect on the mixed tumor, C42B, which is unresponsive to TGFβ. We observed different effects of p38MAP kinase inhibition in these models, which are described below. The p38MAP kinase inhibitor, SD-282 increased bone metastases due to PC-3 prostate cancer and had no effect on LuCAP23.1 or C42B.

Specific Aim 2: To determine the molecular targets of the inhibitors in prostate cancer cells in vitro by gene array analysis (hypothesis 4). The role of an already-identified target of TGFβ, PMEPA1, will be tested in the animal models by overexpressing it in 2 prostate cancer cell lines (hypothesis 5).

Results and Progress: The majority of the work from year 1 has been performed on this aim. Gene array targets of TGFβ on PC-3 prostate cancer were validated by quantitative real-time PCR and were described in the progress report for year one. We are currently performing stable knockdown of PMEPA1 as well as overexpression and plan to test stable cell lines in the bone metastases model. We have also made deletion constructs of the promoter and are in the process of studying the TGFβ regulation of this promoter, which contains a number of Smad binding sites. Finally, we have made mutations of the PPXY sites of PMEPA1, which could bind to inhibitors of the TGFβ pathway. These mutants will be expressed in PC-3 prostate cancer and studied for effects on TGFβ signaling and bone metastases.

Specific Aim 3: To test the efficacy of combined TβRI and p38 MAPK inhibitors against 3 prostate cancer models in vivo (hypothesis 3).

Results and Progress: This aim has not been started and is planned for the last year of the proposal.

Results:

P38MAP kinase inhibitor increases osteolytic bone metastases due to PC-3 prostate cancer. Most recently, we found that treatment with a p38MAP kinase inhibitor, SD-282, accelerated development of osteolytic lesions due to PC-3 prostate cancer (**Figure 1**). In this experiment, mice were treated with SD-282 when bone metastases were identified on radiographs, at 4 weeks. Doses used were based on pharmacokinetic studies done by the manufacturer, Scios, Inc. We found similar results using the osteolytic breast cancer model, MDA-MB-231: p38MAP kinase inhibition increased osteolytic bone metastases. We have ongoing experiments testing this inhibitor on bone metastases due to LuCAP23.1 and C42B. To date, there has been no effect of SD-282 on either group. In this, and in the PC-3 experiment, quantitative bone histomorphometry will be performed.

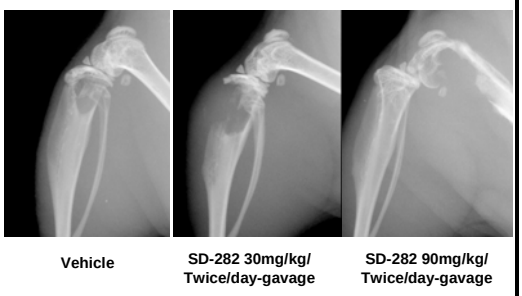

Figure 1: **p38MAPK inhibitor SD-282** and PC-3 bone metastases. Representative radiographs (4X magnification) of legs from mice with PC-3 prostate cancer cells 8wks post tumor inoculation. Larger osteolytic bone lesions in mice treated with SD-282 (right 2 panels) compared to vehicle (left panel).

TGFβRI kinase inhibitor reduces osteolytic bone metastases due to PC-3 prostate cancer. In contrast, and similar to results observed with osteolytic breast cancer model, MDA-MB-231, the TGFβRI kinase inhibitor, SD-208, reduced osteolytic bone metastases and improved survival in mice bearing PC-3 prostate cancers (both treatment and prevention protocols). (**Figure 2, 3**) Taken together with data that a p38MAPK inhibitor was ineffective and even increased PC-3 and MDA-MB-231 bone metastases, it may be better to target the Smad pathway than total TGFβ signaling. The latter may be less effective, if downstream p38MAP kinase blockade adversely affects bone metastases.

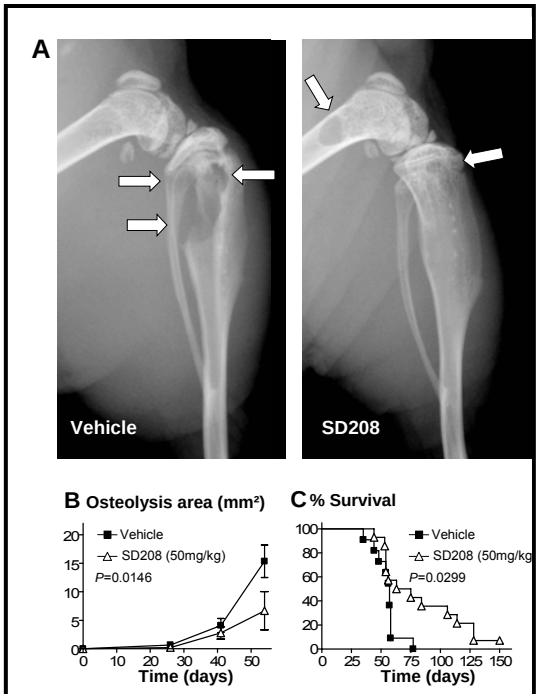

Figure 2. SD-208 prevents PC-3 bone metastases. Mice inoculated i.c. with 10^5 cells PC-3 cells & given 50mg/kg SD-208 (n=14) or vehicle (n=11) 2d prior to inoculation. **A**. X-rays (4X mag) of distal femur & proximal tibia. Arrows point to osteolytic lesions. **B**. Osteolytic lesion area. Ave ± SE, by 2-way ANOVA. **C.** Kaplan-Meier survival curves.

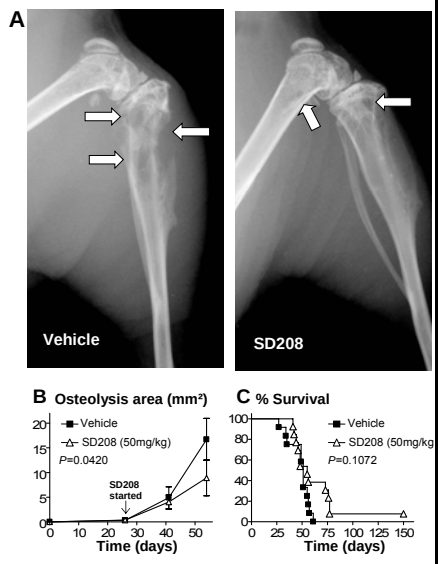

Figure 3: Treatment of established PC-3 bone metastases with SD-208. As in Figure 18, except mice given 50mg/kg SD-208 (n=13) or vehicle (n=12) beginning at d28, when metastases seen on X-ray.

2. TGFβRI kinase inhibitor accelerates osteoblastic bone metastases due to prostate cancer LuCAP23.1. Since our prior studies included only osteolytic bone metastases models, and TGFβ has been implicated in the pathogenesis of prostate cancer metastases to bone, we tested SD-208 in a model of human prostate cancer, LuCAP23.1, which grows as osteoblastic lesions when directly injected into bone. LuCAP23.1 (obtained from our collaborator, Robert Vessella, University of Washington) is an androgen sensitive, PSA-producing human tumor derived from an osteoblastic bone metastasis. It causes osteoblastic lesions in 10-12 weeks. **Figure 4** shows representative radiographs from LuCAP23.1 bearing mice, treated with SD-208 at 20 or 60 mg/kg/day after inoculation into bone. Drug was started at 12 weeks, after lesions were evident by radiographs. The experiment, still in progress, shows clearly that osteoblastic bone metastases are accelerated by treatment with SD-208. These preliminary data indicate that TGFβRI kinase inhibition may worsen osteoblastic metastases. Since our preliminary data also indicate that SD-208 has direct effects to increase osteoblast activity, this host response may accelerate osteoblastic disease in prostate cancer. Since LuCAP23.1 is a xenograft which cannot be studied in culture as a cell line, we will test TGFβ inhibition on other osteoblastic models of breast and prostate cancer. ZR-75-1 is a human breast cancer which causes osteoblastic metastases due to tumor production of ET-1 (Yin et al 2003). We recently derived a variant of the PC-3 prostate cancer line that causes osteoblastic bone metastases, PC-3OB, and are now characterizing it (**Figure 5**). We are also testing SD-208 on bone metastases due to the mixed osteolytic & osteoblastic tumor C42B and, thus far, there has been no effect observed, compared to control. Quantitative histomorphometry will be performed on bones from all experiments.

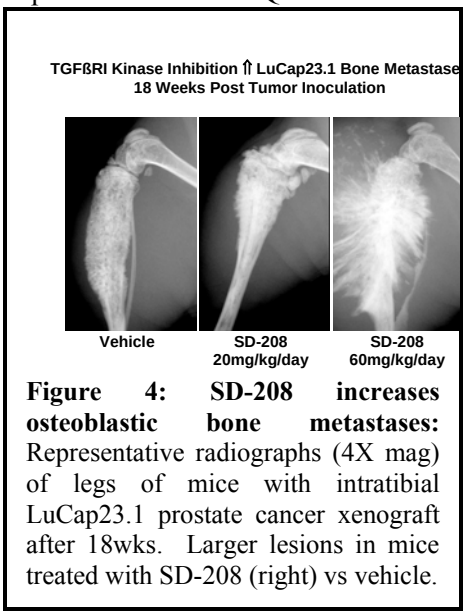

Figure 4: SD-208 increases osteoblastic bone metastases: Representative radiographs (4X mag) of legs of mice with intratibial LuCap23.1 prostate cancer xenograft after 18wks. Larger lesions in mice treated with SD-208 (right) vs vehicle.

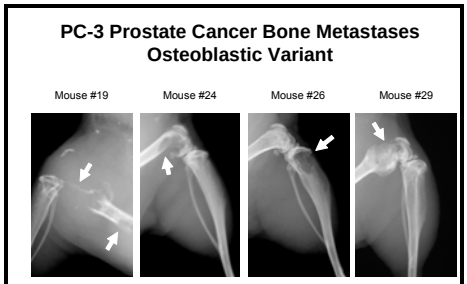

Figure 5: Osteoblastic metastasis in PC-3: Cells inoculated i.c. in 36 mice. One developed osteoblastic lesion (right, mouse #29). Purely osteolytic lesions in mice 19, 24 and 26. New mineralized bone evident in mouse #29. Tumor harvested, cells grown & repassaged.

Reportable Outcomes

Presentations:

- Targeting the endothelin axis in osteoblastic bone metastases: mechanisms and implications. Prostate Cancer Foundation Scientific Retreat, Scottsdale, AZ, Oct 2005.
- What makes bone a favorable site for metastases? AACR Tumor Microenvironment and Protease meeting, Bonita Springs, FL, Dec 2005.
- Bisphosphonate update 2006. 26th Annual Scripps Clinical Hematology and Oncology Meeting, San Diego, CA, Feb 2006.
- TGFβ in bone metastases: implications for therapy. Research Seminar, University of Lyon, Lyon France, Mar 2006.
- TGFβ in bone metastases: pathophysiology to treatment. Research Seminar, INSERM Unit 627, Hospital St. Louis, Paris, France, Mar 2006.
- Gene signatures in bone metastases: Role of TGFβ. European Calcified Tissue Society Meeting, Prague, Czech Republic, May 2006.
- TGFβ in skeletal complications of malignancy. Seminar at Schering, Berlin, Germany, May 2006.
- Osteoporosis in the cancer patient. First international meeting on secondary causes of osteoporosis, Florence, Italy, Jul 2006.
- PTHrP in osteoblastic bone metastases. PPP meeting. Newfoundland, Canada, Jul 2006.
- Pathophysiology of metastases. Regional Medical Liaison meeting, UCSF, San Francisco, CA, Jul 2006.
- Bone micrometastases. International Breast Cancer Conference. Kona, HI, Aug 2006.
- Pathophysiology of Bone Metastases. International Metastases Meeting, Tokushima, Japan, Sep 2006.
- TGFβ in bone metastases. Animal Model Working Group, American Society for Bone and Mineral Research Meeting, Philadelphia, PA, Sep 2006.
- Molecular mechanisms of bone metastases: Role of TGFβ. Fat and Bone Meeting, Madrid, Spain, Sep 2006.
- Molecular mechanisms of bone metastases: osteolytic and osteoblastic. Italian Cancer Society Meeting, Bari, Italy, Oct 2006.
- TGFβ in bone metastases: pathophysiology to treatment. Visiting Professor, University of Minnesota, Dec 2006.
- TGFβ signaling in breast cancer bone metastases: friend or foe? Cancer and Bone Society Meeting, San Antonio, TX Dec 2006.
- Molecular mechanisms of bone metastases: insight into therapy. Endocrine Grand Rounds, University of Texas Health Science Center at San Antonio, TX, Dec 2006.
- Skeletal complications of cancer and cancer treatment. Bone Club, San Antonio, TX, Dec 2006.
- Skeletal health in the cancer patient. Maine State Osteoporosis Meeting, Sugarloaf, MN, Jan 2007.
- Effects of bisphosphonates on tumor cells. Consensus on Bone Loss in Cancer Patients on Aromatase Inhibitors. Geneva, Switzerland, February, 2007.
- TGFβ in bone metastases: pathophysiology to treatment. Institute for Molecular Medicine, University of Lisbon, Lisbon, Portugal, March 2007
- RANK Ligand in prostate cancer metastases to bone. Medical Grand Rounds, Hospital Santa Ana, University of Lisbon, Lisbon, Portugal, March 2007
- Skeletal health in the cancer patient. Endocrine Grand Rounds, Oregon Health Sciences University, Portland, OR. April 2007

- TGFβ signaling in breast cancer bone metastases: friend or foe? Research Seminar, Oregon Health Sciences University, Portland, OR. April 2007
- TGFβ in bone metastases: pathophysiology to treatment. Endocrinology Grand Rounds, Mount Sinai School of Medicine, New York, NY, April 2007
- TGFβ signaling in breast cancer bone metastases: friend or foe? Advances in Mineral Metabolism Meeting, Snowmass, CO, April, 2007
- TGFβ signaling in cancer metastases to bone: friend or foe? Cleveland Clinic, April, 2007
- Biology of bone metastases. FASEB Meeting, Washington, D.C., April 2007
- Endothelins in pathologic and normal bone remodeling. Bone Club. University of Pittsburgh, Pittsburgh, PA, May 2007
- Endothelins in pathologic and normal bone remodeling. Research Seminar, Wyeth, Collegeville, PA. May 2007

Publications

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Conclusions

A central tenet of the field of bone metastases is that the bone microenvironment supplies factors, such as TGF-β, stimulating prostate cancer cell signaling and altering their phenotype. TGF-β signaling in cancer is however complex and can lead to the activation of numerous genes. We have identified many of these genes by microarray analysis and have validated the gene reported here. Of these, PMEPA1 as the most highly upregulated gene. We have cloned the PMEPA1 promoter and full-length gene and have begun promoter analysis of the TGFβ response element. We are in the process of overexpressing and silencing PMEPA1 in prostate cancer lines.

In vivo experiments to determine the effect of a TGFβ RI kinase inhibitor, SD-208, on the development and progression of prostate cancer metastases to bone due to PC-3, LuCAP and C42B prostate cancers have shown differential effects, depending on the radiographic phenotype of the bone metastases. SD-208 improves osteolytic bone metastases due to PC-3, but increases osteoblastic bone metastases due to LuCAP23.1 and has no effect on mixed C42B lesion. The results in C42B are not surprising, given the fact that this line is unresponsive to TGFβ. However, the effect of this compound to increase osteoblastic bone metastases is a significant concern. This needs to be confirmed the information utilized with respect to design of future clinical trials. We have initiated an agreement with Eli Lilly to study another TGFβRI kinase inhibitor which is currently in clinical trials for patients with bone metastases due to all solid tumors.

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